Cigarette smoking represses expression of cytokine IL-12 and its regulator miR-21 - an observational study in patients with coronary artery disease

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ABSTRACT

Rationale

The heterodimer IL-12 is an inducer of Th1 responses and stimulates INFγ production. Micro-RNA-21 (miR-21) is described as a key regulator of the pro-inflammatory response and has IL-12p35 mRNA as one of its main targets. The IL-12p40 1188A/C genetic variant located in 3'untranslated region (UTR), thus environmentally exposed, has further been reported to modify IL-12 levels. We have previously reported on the lowering effect of cigarette smoke on circulating IL-12 in patients with coronary artery disease (CAD).

Objectives

To explore if cigarette smoking affects IL-12p35, IL-12p40, INFγ and miR-21 gene-expression and further modulates any effect of the IL-12p40 polymorphism on circulating IL-12 levels.

Methods and Results

The IL-12p40 1188A/C polymorphism was analyzed in 1001 stable CAD patients, of which 330 subjects were included for IL-12p35, IL-12p40 and INFγ gene-expression analyses in circulating leukocytes and 200 were further selected for plasma miR-21 analysis.

Smoking associated with lower expression of miR-21 and its target IL-12p35 mRNA (adjusted $p<0.05$, both) whereas the influence on INFγ expression tended to be high-dose reliant ($p=0.057$). The IL-12p40 CC genotype associated with elevated circulating IL-12 levels, however, when stratified according to smoking, only in the non-smoking group (adjusted $p<0.05$).

Although the markers were mainly downregulated in current smokers, their inter-correlations were potentiated.

Conclusion

Smoking associated with reduced miR-21 gene-repression and the results can therefore not explain the previously observed reduction in circulating IL-12. Smoking attenuated the IL-12 pro-inflammatory axis in which the investigated IL-12p40 genetic variant may have different clinical impact in smokers vs non-smokers.

Keywords:

MiR-21, IL-12, INFγ, cigarette smoking, gene-expression, genetic polymorphism

Non-standard Abbreviations and Acronyms:

ath-159a	Arabidopsis thaliana-159a
ASCET	Aspirin non-responsiveness and Clopidogrel Endpoint Trial
CSE	cigarette smoke extract
Ct	cycle threshold
EDTA	ethylenediaminetetraacetic acid
HWE	Hardy-Weinberg equilibrium
let-7	Caenorhabditis elegans-7
miR	micro-RNA
PCR	polymerase chain reaction
PDCD4	Programmed Cell Death 4
snRNA	small nuclear RNA
RNU	small nucleolar RNA
RQ	relative quantification
Th	T-helper cell
UTR	untranslated region

INTRODUCTION

The pro-inflammatory cytokine interleukin (IL)-12 is critical in the initiation and progression of the Th-1 response, characterized by production of interferon gamma (INFγ) by different T-cells and natural killer cells [1]. IL-12 secretion occurs early in the immune response and seems to play a key role in the regulation of innate and adaptive immunity and to mediate anti-tumor activity [1;2]. The heterodimer IL-12p70 (70 kilo Daltons (kDa)), henceforth referred to as IL-12, is composed of two disulfide-linked polypeptide chains, IL-12p35 (35 kDa) and IL-12p40 (40 kDa), synthesized from two differently located genes, which are transcriptionally and independently regulated [3]. IL-12 is mainly produced by B-cells, monocytes, macrophages and dendritic cells (DCs) and the release of bioactive IL-12 is thought to require that both subunits are expressed and further dimerized within the same cell [4].

We have previously reported on the substantial lowering effect of cigarette smoking on circulating IL-12 levels in patients with coronary artery disease (CAD) [5]. Similar effects have been observed in bronchoalveolar lavage and gingival crevicular fluid of current smokers [6;7] In vitro studies have reported decreased IL-12p40 mRNA levels in macrophages exposed to cigarette smoke extract (CSE), probably mediated by enhanced levels of the p40 transcriptional repressor c-fos [8]. Independent of cell-type or species origin, exposure of CSE has further been described in *in vitro* studies to modify INFγ expression and to impair INFγ signaling, although contradictory reported [9-12].

MicroRNAs (miRNAs) are naturally occurring short non-coding RNA molecules that regulate the expression of target genes post-transcriptionally, by messenger RNA (mRNA) degradation and/or translational inhibition. MiRNAs can be released by cells into the circulation in association with micro-particles, exosomes, or specific proteins, thus serving as potential biomarkers. MiRNA-21 (MiR-21) is the most abundantly expressed small non-coding RNA in multiple mammalian cells, including hematopoietic cells of the immune system, like B- and T-cells, monocytes, macrophages and DCs. It is upregulated in many disease states as cardiac injury, multiple neoplasms and inflamed tissue [13]. Targets of miR-21 are mainly tumor suppressor genes, including the IL-12p35 gene, in which a functional, conserved miR-21 binding site in the 3'untranslated region (UTR) has been described [14]. Repression of IL-12p35 expression by miR-21 could therefore lead to less production of IL-12. MiR-21 has further been defined as

the major regulator of Th1 versus Th2 responses, thus influencing the IL-12/INFγ pro-inflammatory pathway [15].

Genetic variability of the IL-12 genes may also influence the extent of IL-12 production. Many studies have focused on the polymorphic site at position 1188 in the 3' UTR of the IL-12p40 gene, which was reported to increase *in vitro* IL-12 secretion rather than to increase levels of the single IL-12p40 subunit [16]. The 1188 C-allele, also associated with decreased IL-12p40 serum levels [17], has been reported to increase the overall risk of cancers [18] and was further associated with increased risk of bladder cancer in cigarette smokers [19] indicating a IL-12p40 gene-environment interaction.

According to our previous findings of low IL-12 levels in smokers, we aimed in the present study to investigate the influence of cigarette smoke on markers involved in the IL-12/INFγ pro-inflammatory axis, including circulating miR-21 and IL-12p35, IL-12p40 and INF-γ gene-expression in circulating leukocytes, in patients with stable CAD. The IL-12p40 1188 A/C polymorphism was further analyzed according to IL-12 levels and smoking habits. We hypothesized an up-regulation of miR-21 and repression of the IL-12 and INFγ genes in current smokers, with potential influence of the IL-12 p40 genetic variant.

MATERIALS and METHODS

Subjects

The present investigation is a sub-study of the ASCET trial in Norway [20 21], which included 1001 patients with stable CAD (97% Caucasians, 78% men, 20 % current smokers, mean age 62 year) in the time period from 2003-2008. Smoking habits were recorded in 4 categories: 0; non-smokers, 1; quitted smoking > 3 months ago, 2; current smokers ≤ 10 cigarettes per day, 3; current smokers > 10 cigarettes per day. The data analyses are mainly based on differences between non-smokers (0 and 1) and current smokers (2 and 3, n = 201 in the total cohort), if not otherwise stated. Genotyping of the IL-12p40 1188 A/C variant was performed in all patients, whereas 330 subjects (selected between the 450 first randomized patients) were included in the IL-12p35, IL-12p40 and INFγ gene-expression analyses, of which 200 patients were selected for miR-21 analysis, to equalize numbers of smokers and non-smokers.

Ethics Statement

The ASCET study was performed according to the Declaration of Helsinki and approved by The Regional Committee of Medical Research Ethics in South-Eastern Norway. All subjects gave their written informed consent to participate. The ASCET trial is registered at clinicaltrials.gov, with the identification number NCT00222261.

Blood Sampling and standard biochemical analyses

In fasting condition, between 8.00-10.00 a.m., blood samples were collected at entrance into the ASCET study. Routine analyses were performed by conventional methods. Ethylenediaminetetraacetic acid (EDTA) plasma was prepared by centrifugation within 30 minutes, at 2500 x g at 4°C for 20 minutes for plasma miRNA isolation. EDTA blood and PAXGene tubes were collected for DNA and total RNA extraction, respectively. All material was kept frozen at -80°C until further processing and analysis.

Isolation procedures

Prior to the isolation of miRNAs, EDTA plasma was additionally processed with an extra centrifugation step at 16.000 x g for 4°C in 5 minutes. MiRNAs were subsequently isolated from the supernatant of 200 samples, 100 current smokers and 100 non-smokers matched by age and gender, by use of magnetic beads and the TaqMan miRNA ABC Purification kit, Panel A (Applied Biosystems by Life Technologies, Foster City, CA, USA). Prior to miRNA purification, all samples were spiked with 2 µl of 1 nM Arabidopsis thaliana miR-159a (ath-miR159a) according to manufacturers' protocol, to control for differences in recovery during the isolation procedure and further amplification efficiency. Briefly, 100 µl lysis buffer was added to 50 µl plasma and the miRNAs were hybridized to the magnetic beads and further eluted in 100 µl elution buffer and stored at -80°C until analysis. The Panel A of the purification kit isolates 377 specific miRNAs, including miR-21 and the selected reference genes.
Total RNA was extracted from 330 samples, using PAXGene Blood RNA kit (PreAnalytix, Qiagen, Germany), with an extra cleaning step (RNeasy MinElute Cleanup Kit, Qiagen). Circulating leukocytes were hence the source for RNA. Briefly, RNA was extracted using ethanol and spin columns and eluted in 14 µl elution buffer and stored at -80°C until analysis.
DNA was automatically isolated from 1001 EDTA whole-blood samples by the MagNA Pure LC DNA Isolation kit on the MagNA Pure LC instrument (Roche Diagnostics, Germany). In brief, DNA was isolated from 500 µl whole-blood by magnetic beads and eluted in 100 µl elution buffer and stored at -80°C until analysis.

Concentration and purity of extracted RNA and DNA were assessed using NanoDrop 1000 spectrophotometry (NanoDrop Technologies, DE, USA).

Reference genes tested for normalization in the miR-21 analysis

To test for suitable reference genes in the miR-21 analysis, 7 different miRNAs were validated in initial experiments in 12 smokers and 12 non-smokers collected from the same cohort: U6 small nuclear RNA (U6 snRNA), snRNA+small nucleolar RNA (RNU) 44, RNU 48, miR-425-5p, miR-16 -5p, Caenorhabditis elegans (let) -7a-5p and let-7g-5p (Assay ID: 001973, 001094, 001006, 001516, 000391, 000377, 002282, respectively, Applied Biosystems), The tested miRNAs were chosen based on manufactures recommendations and reports from the literature [22-26] U6 snRNA, RNU 44 and RNU 48 were not expressed in our test samples. Due to higher observed cycle threshold (Ct) mean for let-7a and let7g (34.2 and 33.3, respectively), as compared to miR-425 (32.7) and miR-16 (25.8), and less stable expressed miR-16 as compared to miR-425 (SD 1.14, correlation of variation (CV) 4.4% vs. SD 0.96, CV 2.4%, respectively), mir-425 was chosen as the reference gene to normalize miR-21 expression. Additionally, for this validation experiment, no influence on expression by cigarette smoke was essential for the choice.

IL-12 p40 genotyping

Allelic discrimination of the polymorphism IL-12p40 3'UTR 1188 A/C (rs3212227, assay ID C-2084293_10) was performed on the VIIa 7 instrument with TaqMan Genotyping Master Mix Part. No. 4371357 (Applied Biosystems), and 1 µl of genomic DNA (1-20 ng) in a final reaction volume of 25 µl. Genotype calling was automatically applied in the assay, with 95% confidence. About 5% of the samples were reanalyzed, with 100 % concordance.

Relative real-time PCR quantification of miRNAs and mRNAs

Expression of all genes was measured on the VIIa 7 instrument using TaqMan Universal polymerase chain reaction (PCR) Master Mix, No AmpErase UNG and TaqMan assays (Applied Biosystems), as relative quantification (RQ) ($2^{-\Delta\Delta Ct}$ method) [27].

The spike-in ath-miR-159a in combination with miR-425 was used as endogenous controls for normalization of miR-21 expression. Reverse transcription and expression analysis of miR-21, miR-425 and ath-miR-159a was performed and analyzed using the TaqMan MicroRNA Reverse Transcription kit with the application of 5 µl miRNA, and the miR assays (IDs: 000397, 001516,

000338, respectively). The miR assays contain primers for each individual miR for the reverse transcription step and specific primers and probes for the PCR step.

Total RNA (100 ng) was reversely transcribed into complementary DNA (cDNA) by use of qScript cDNA SuperMix, using oligo (dTs) and random primers (Quanta Biosciences, Inc., Gaithersburg, USA). Expression of the IL-12p35 (Hs01073447_m1), IL-12p40 (Hs01011518_m1) and INFγ (Hs00989291_m1) genes were normalized to β-2 macroglobulin (Hs99999907_m1) expression levels, previously tested as a valid house-keeping gene in this population [28].

The PCR reaction for miRNAs and mRNA targets were carried out in volumes of 20 µl, with application of 1.33 µl and 2 µl cDNA, respectively. Individual amplification curves for all assays were carefully validated. Samples with Ct values >35 were not included in the data analysis, and template-negative controls were included in each run.

Statistics

As the measured variables were unequally distributed, non-parametric tests were applied. For group comparisons, Kruskal-Wallis and Mann-Whitney tests, when appropriate, were used for continuous data and the χ^2 test for categorical data. The associations between the markers and cigarette smoking were analyzed by linear regression using log transformed data. The models were adjusted for age, gender and C-reactive protein (CRP) in miR-21 association (cf. Table 1), further total cholesterol and the presence of hypertension in gene-expression associations (cf. Online Supplementary Table 1), and additionally triglycerides and high-density lipoprotein cholesterol in the genotype associations with circulating IL-12 levels. As the use of angiotensin II receptor blocker and insulin were differently distributed between non-smokers and smokers in the total cohort (cf. Online Supplementary Table 2), the use of these medications was further adjusted for in all regression models. Spearman's Rho was used for correlation analysis. The Hardy-Weinberg equilibrium (HWE) was tested for the IL-12p40 polymorphism using the χ^2 test. All statistical analyses were performed by SPSS 21.0 (SPSS Inc., Chicago, Illinois, USA). A two-tailed probability test of 0.05 or less was considered statistically significant.

RESULTS

Demographic data in the miR-21 cohort (n = 200) according to cigarette smoking are presented in Table 1, showing similar distribution of the variables between current smokers and non-smokers, except of higher CRP values in the smoking group. We have previously reported on the lowering effect of smoking on circulating IL-12 in the total ASCET population (n = 1001) [5]. In the present miR-21 cohort, circulating IL-12 was similarly and significantly lower (43%) in current smokers vs. non-smokers (51 pg/mL vs.89 pg/mL, adjusted p-value < 0.001).

MiR-21 expression

The isolation and analysis of miR-21 was successfully performed in plasma samples from 196 patients (99 current smokers). Four samples were excluded due to miR-21 Ct values >35. Based on the initial experiments, miR-425 and the spike-in ath-miR-159a control were used as reference genes for normalization. MiR-425 was analyzed in 196 subjects and was not differently expressed in smokers vs. non-smokers (p = 0.9). The spike ath-miR159a was successfully recovered in 199 samples (Ct mean = 17.2 with CV and SD of 2.15 % and 0.37, respectively).

Cigarette smoking associated with 16% lower expression of miR-21 (p = 0.011, adjusted) (Figure1A). However, at particular low circulating IL-12 levels (< 37 pg/mL; 25 percentile in current smokers), miR-21 was significantly upregulated in current smokers (p = 0.046, adjusted) (Figure 1B), with highest RQ levels in the group of heavy smokers (>10 cigarettes pr. day, data not shown).

Gene-expression of IL-12p35, IL-12p40 and INFγ

Expression of the genes in circulating leukocytes was analyzed in 330 patients (96 current smokers). Demographic data in this cohort are shown in Online Supplement Table 1. IL-12p35 was significantly downregulated in current smokers (p = 0.014, adjusted) (Figure 2A), whereas expression of IL-12p40 mRNA was not detectable. In patients with the particularly low circulating IL-12 levels, IL-12p35 mRNA levels were observed additionally reduced in current smokers and especially in heavy smokers (p = 0.045, adjusted) (Figure 2B).

Expression of INFγ was not differently expressed between smokers and non-smokers and also not between the 4 smoking groups (p >2.0, both), however, a borderline reduction in INFγ mRNA levels was observed in heavy smokers (category 3) vs. smokers in category 2 (p = 0.057) (Figure 2C).

Genotyping of the IL-12p40 polymorphism

The IL-12 p40 1188A/C polymorphism was successfully analyzed in 992 subjects and the AA (n = 651), AC (n = 309) and CC (n = 32) genotype distribution was in HWE. Demographic data in the total ASCET cohort are shown in Online Supplementary Table 2. The observed minor allele frequency of 0.19 is in line with previous reports performed in Caucasians [29]. The achieved allelic discrimination data were investigated according to circulating IL-12 levels and cigarette smoking in the total ASCET cohort. The CC genotype associated with significantly higher IL-12 levels as compared to A-allele carriers (p = 0.006, adjusted) (Figure 3A). When stratified according to smoking or not, this association was only present in the non-smoking group (p = 0.012, adjusted) (Figure 3B).

Association analyses

We observed a weak but significant correlation between miR-21 and circulating IL-12 (r = 0.22, p = 0.002, n = 196), whereas miR-21 was not significantly correlated to expression of the IL-12 p35 and INFγ genes. Circulating IL-12 levels correlated weakly to RQ values of the IL-12 p35 gene (r = 0.13, p = 0.016, n = 331), however, stronger correlated in current smokers (r = 0.32, p = 0.001, n = 96), in which expression of the IL-12p35 and INFγ genes inter-correlated (r = 0.27, p = 0.007, n = 96). No significant correlations between these variables were observed in non-smokers. Moreover, miR-21 and CRP were significantly correlated (r = 0.195, p = 0.006) and strengthened in the non-smoking group (r = 0.36, p<0.001, n = 97).

We also observed that angiotensin II receptor blocker and insulin significantly induced elevated levels of circulating IL-12 and mir-21 (only insulin) (p<0.05, both, data not shown).

Summary of results

A schematic illustration of the overall achieved results is presented in Figure 4.

Table 1 Demographic data present in the cohort analyzed for miR-21 as related to cigarette smoking

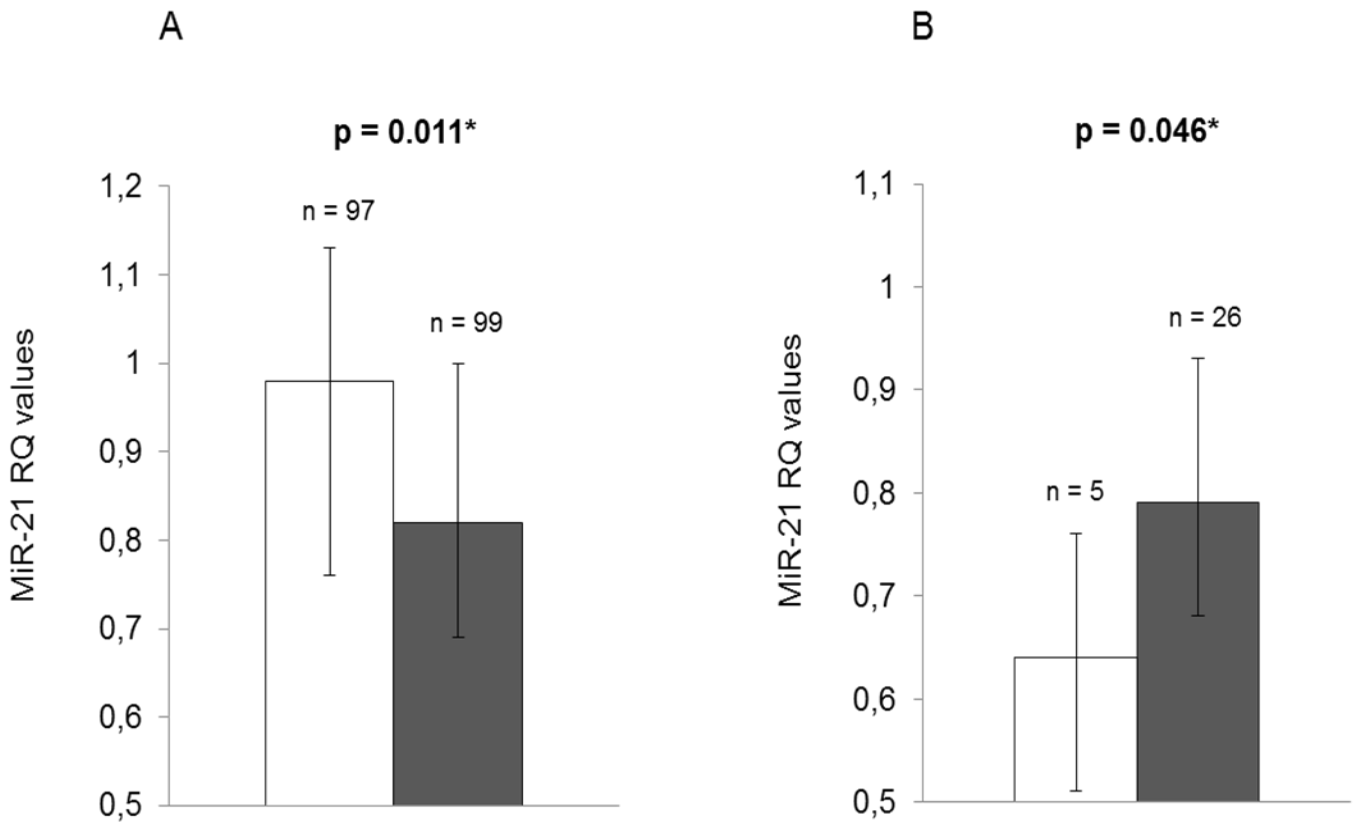
	Non-smokers (n = 100)	Current smokers (n = 100)	p-value
Age (years, mean (range))	59 (37-75)	58 (39-75)	ns
Men/Women n (%)	77/20 (79/21)	76/23 (77/23)	ns
Type 2 Diabetes n (%)	24 (25)	19 (19)	ns
Hypertension n (%)	54 (56)	43 (43)	ns
BMI ($kg/m^{2)*}$	27.0 (25.2, 30.0)	27.0 (24.4, 30.4)	ns
CRP (mg/L)*	1.80 (0.90, 3.00)	2.5 (1.50, 4.30)	**0.003**
Triglycerides (mmol/L)*	1.60 (0.96, 2.05)	1.49 (1.07, 1.96)	ns
Tot-C (mmol/L)	4,7 (1.0)	4.9 (1.1)	ns
HDL-C (mmol/L)	1.3 (0.5)	1.3 (0.4)	ns
Fasting glucose (mmol/L)	6.6 (2.5)	6.2 (2.1)	ns

Values are mean (SD) or number (proportions) if not otherwise stated. SD: standard deviation, BMI: body mass index, CRP: C-reactive protein, Tot-C: total cholesterol, HDL-C: high-density lipoprotein cholesterol.

p-values refer to differences in variables between non-smokers and current smokers

* Median levels (25, 75 percentiles)

Figure 1


A
p = 0.011*
MiR-21 RQ values
1,2
1,1
1
0,9
0,8
0,7
0,6
0,5
n = 97
n = 99
B
p = 0.046*
MiR-21 RQ values
1,1
1
0,9
0,8
0,7
0,6
0,5
n = 5
n = 26

Legend to Figure1: Expression of miR-21

Expression of miR-21 as related to cigarette smoking in the total miR-21 cohort (A), and in the subgroup of subjects presenting with low IL-12 levels (< 37 pg/mL; 25 percentile in current smokers) (B).

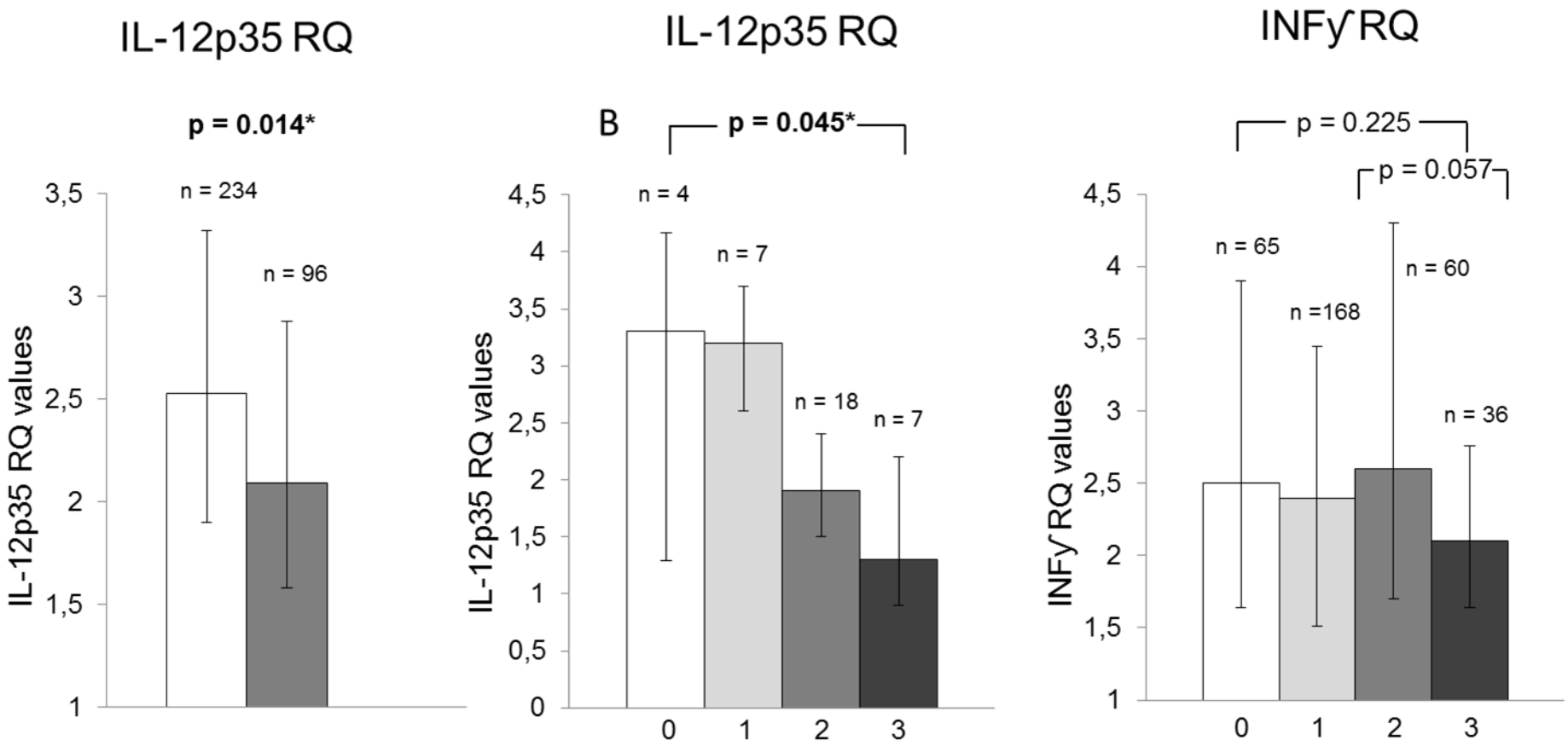
White bars; non-smokers, grey bars; current smokers

RQ: relative quantification

p-values refer to differences in relative expression of miR-21 between non-smokers and current smokers (*adjusted for age, sex, C-reactive protein, use of angiotensin II receptor blocker and insulin).

Error bars represent the 25- and 75 percentiles.

Figure 2


IL-12p35 RQ
p = 0.014*
IL-12p35 RQ values
3,5
3
2,5
2
1,5
1
n = 234
n = 96
IL-12p35 RQ
B
p = 0.045*
IL-12p35 RQ values
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
n = 4
n = 7
n = 18
n = 7
0
1
2
3
INFγ RQ
p = 0.225
p = 0.057
INFγ RQ values
4,5
4
3,5
3
2,5
2
1,5
1
n = 65
n =168
n = 60
n = 36
0
1
2
3

Legend to Figure 2: Gene-expression of IL-12p35 and INFγ

Expression of the IL-12p35 gene as related to cigarette smoking in the total gene-expression cohort (A). White bars; non-smokers, grey bars; current smokers.

Expression of the IL-12p35 gene in the subgroup of subjects presenting with low IL-12 levels (<37.4 pg/mL; 25 percentile in current smokers), as related to degree of smoking (B). White, and grey bars refer to degree of smoking divided in 4 groups: white; non-smokers, light grey; quitted smoking > 3 months ago, grey; current smokers ≤ 10 cigarettes per day, dark grey; current smokers > 10 cigarettes per day.

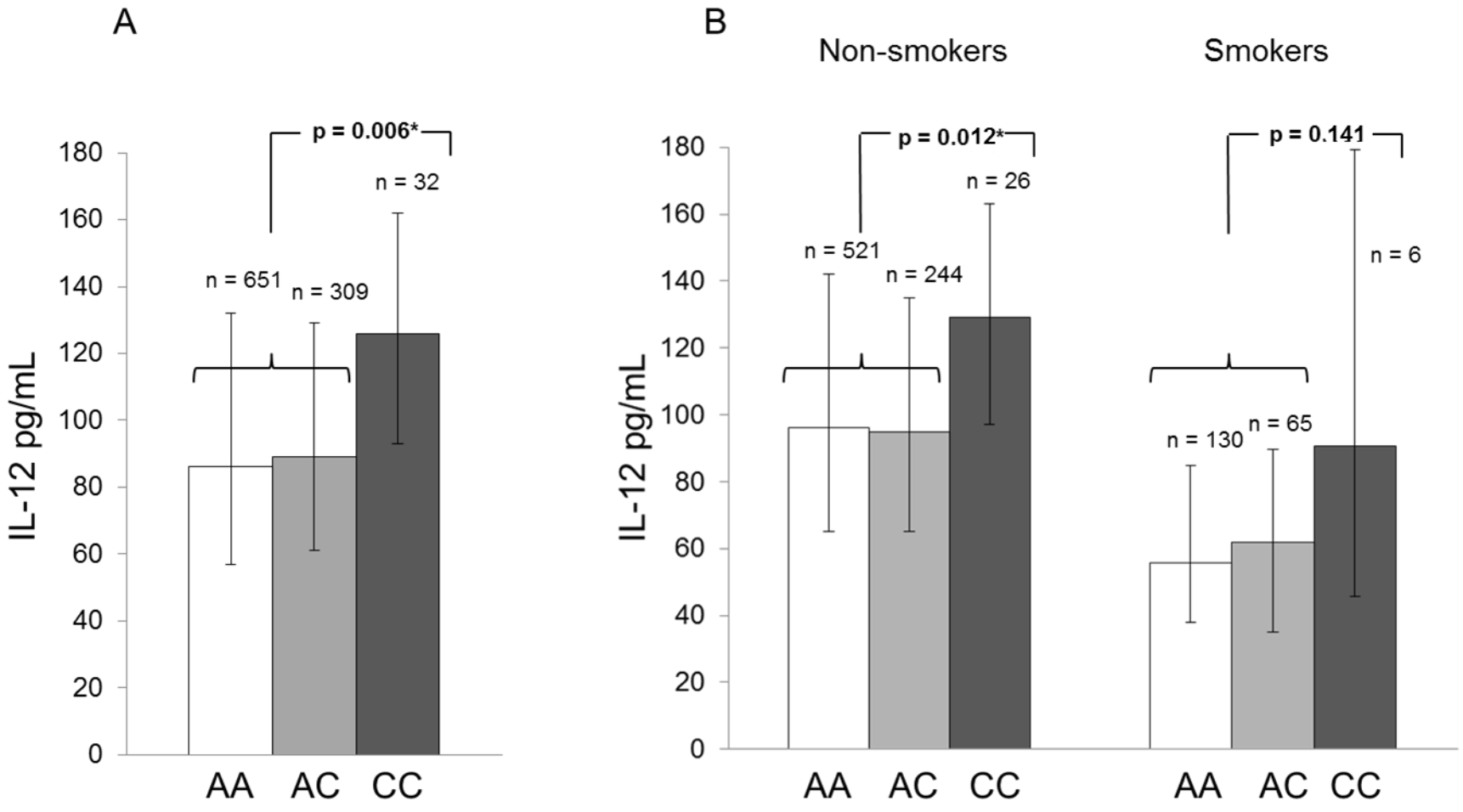
Expression of INFγ, as related to degree of smoking in the total gene-expression cohort (C). White, and grey bars refer to degree of smoking divided in 4 groups: white; non-smokers, light grey; quitted smoking > 3 months ago, grey; current smokers ≤ 10 cigarettes per day, dark grey; current smokers > 10 cigarettes per day.

RQ: relative quantification

p-values refer to differences in relative expression of the IL-12p35 and INFγ genes, as related to smoking groups (*adjusted for age, sex, the presence of hypertension, C-reactive protein, total cholesterol, use of angiotensin II receptor blocker and insulin).

Error bars represent the 25- and 75 percentiles.

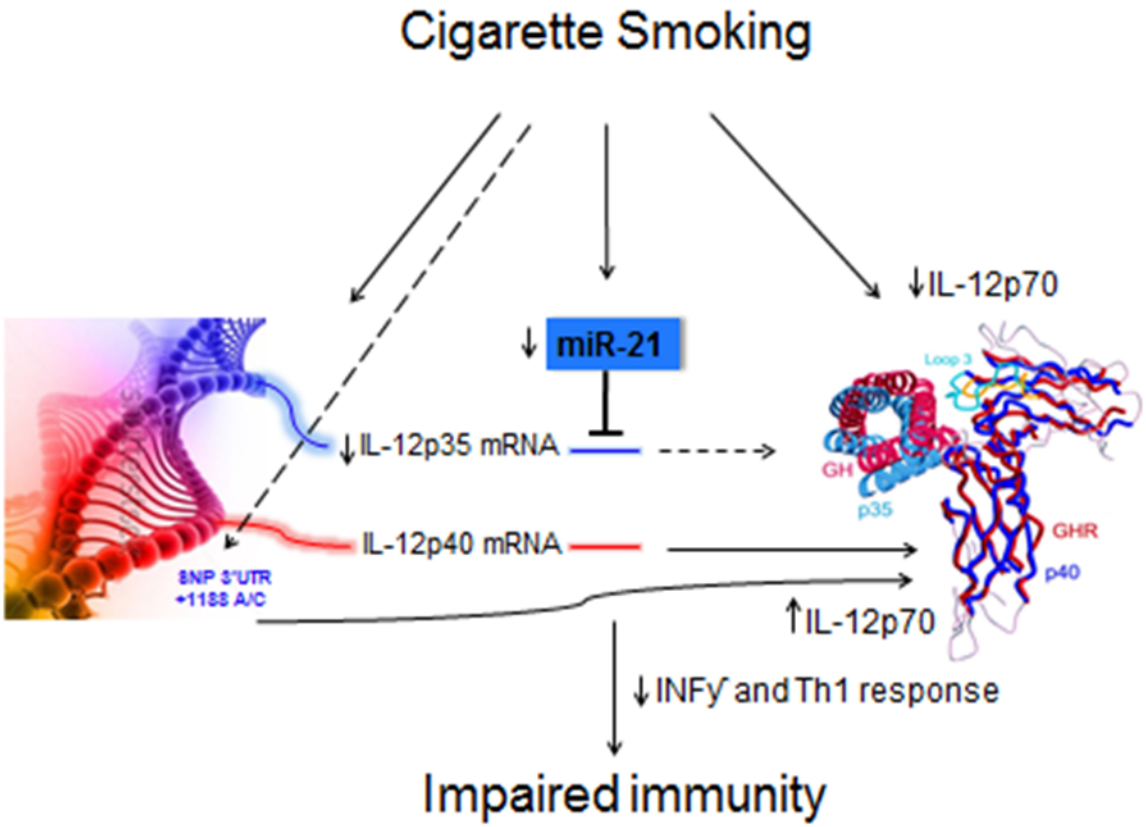
Figure 3


A
B
Non-smokers
Smokers
p = 0.006*
p = 0.012*
p = 0.141
n = 651
n = 309
n = 32
n = 521
n = 244
n = 26
n = 130
n = 65
n = 6
IL-12 pg/mL
0
20
40
60
80
100
120
140
160
180
AA
AC
CC

Legend to figure 3: Influence of the IL-12p40 1188 A/C polymorphism on circulating IL-12

Influence of the IL-12p40 1188 A/C polymorphism on circulating IL-12 levels in the total cohort (A) and stratified according to smoking/non-smoking (B). White, light grey and dark grey bars refer to AA, AC and CC genotypes.
p-values refer to differences in IL-12 levels between CC and AA/AC genotypes (adjusted for age, sex, the presence of hypertension, C-reactive protein, triglycerides, total cholesterol, high-density lipoprotein cholesterol, use of angiotensin II receptor blocker and insulin).
Error bars represent the 25- and 75 percentiles.

Figure 4


Cigarette Smoking
↓IL-12p70
↓ miR-21
↓IL-12p35 mRNA
IL-12p40 mRNA
SNP 3'UTR
+1188 A/C
Loop 3
GH
p35
GHR
p40
↑IL-12p70
↓INFγ and Th1 response
Impaired immunity

Legend to Figure 4: Effects of cigarette smoking on immune regulation

Schematic illustration on the effects of smoking on miR-21expression and the IL-12/INFγ pro-inflammatory axis and further influence of the IL-12p40 1188A/C polymorphism on levels of the IL-12 heterodimer.

Full-line arrows indicate the observed significant associations between cigarette smoking and 1) Reduced expression of the IL-12 p35 gene, 2) Reduced expression of miR-21, 3) Reduced circulating levels of the heterodimer IL-12.

Full-lines indicate further the observed significant association between the IL-12p40 +1188 A/C polymorphism and elevated circulating levels of the heterodimer IL-12.

The broken-line arrows indicate that 1) It is not clear wether cigarette smoking affects the influence of the IL-12p40 +1188 A/C polymorphism on circulating IL-12 levels and 2) Our results on the influence of cigarette smoking on miR-21 cannot explain the lower circulating levels of the heterodimer IL-12.

In total, cigarette smoking seems to attenuate the pro-inflammatory axis, including INFγ and the TH1-cell response, which partly may explain why smokers have impaired immunity. The observed effects may be modulated by the IL-12p40+1188A/C polymorphism.

SNP; single nucleotide polymorphism

DISCUSSION

Our hypothesis of increased miR-21 expression in current smokers could have explained the previously reported lower circulating IL-12 levels in these subjects. However, cigarette smoking was significantly associated with lower expression of miR-21 (Figure 1A) and its target IL-12p35 mRNA (Figure 2 A) in the present study. Hence, our miR-21 results cannot clarify the pronounced reduction in circulating IL-12 in current smokers, although in a small group of patients with particular low IL-12 levels, miR-21 was significantly upregulated in smokers (Figure 1B), with corresponding additionally lower IL-12p35mRNA levels (Figure 2B). Other unknown/unmeasured miR-21 co-stimulating factors/regulators in these subjects might thus be suggested. INFγ was not significantly affected by smoking in general, however, a tendency of repressed INFγ gene-expression related to degree of smoking was observed (Figure 2C).

Modified expression of miR-21 has been described as related to CSE exposure and tobacco use in experimental and human studies, however contradictory reported [30]. The discrepancy may be due to type and duration of exposure. Differences in analytical models, i.e. choice of normalization gene (s) and/or cellular contamination and hemolysis of plasma (and serum) may additionally have contributed to lack of consistency between results, including the present investigation. However, in the initial experiments for this study, expression of miR-21 was observed numerically lower expressed in current smokers independently of normalization against miR-425, mir-16, the spike ath-159a or combinations of multiple endogenous controls. Moreover, hemolytic plasma samples were not utilized for miRNA isolation, and in an attempt of diminishing cellular debris, an additional centrifugation step was performed prior to isolation. The spike-in ath-mir-159a added to all 200 samples before the isolation procedure, controls additionally for the isolation procedure, and further processing with the conversion of miRNAs to cDNA, and PCR efficiency. The applied spike control in the present investigation was successfully recovered in 99.5% of the samples.

Influence of the studied IL-12p40 polymorphism on circulating IL-12 levels was especially present in non-smokers (Figure 3B). Lack of similar significant increase in IL-12 with increasing number of C-alleles in smokers, may probably be due to the low number with respective genotypes. Interestingly, IL-12 levels associating with the CC genotype in smokers were similar to IL-12 levels in non-smoking A-allele carriers (Figure 3B). In atherosclerosis, it

might thus be suggested a beneficial AA/AC genotype in non-smokers, and further a superior CC genotype in smokers as related to IL-12' role in immunity and anti-tumor activity. The latter has, however, been contradictory reported in a smaller study on the risk of bladder cancer [19].

A positive correlation was observed between circulating IL-12 and expression of miR-21. MiR-21 was not correlated to IL-12p35-mRNA, however, parallelly altered in current smokers. Thus, our results may indicate miR-21 to be a modest repressor of IL-12p35mRNA, as IL-12 and miR-21 expression seem to mirror each other rather than being inversely correlated. The IL-12/INFγ pro-inflammatory axis, in which smoking seems to potentiate the interplay between the investigated markers, may involve regulatory mechanisms additional to miR-21.
MiR-21 was, in addition to smoking habit, observed correlated to CRP, supporting the previously described association between miR-21 and inflammation [31].

The low IL-12 levels observed in smokers may have clinical implications concerning both inflammation and malignancies. The impaired immunity described in airways of current smokers may partly be addressed by the observed attenuated IL-12 gene- and protein expression in the circulation, and further the reported IL-12 modifications in experimental studies [6;7]. IL-12 has emerged as one of the most potent cytokines in mediating antitumor activity [32]. Bio-informatically predicted targets for miR-21 are mainly tumor suppressor genes, including IL-12p35 and further also the protein entitled Programmed Cell Death 4 (PDCD4). Loss of PDCD4 has been associated with increased tumor aggressiveness [33], and PDCD4 was similarly reported to be lower expressed in current smokers [34]. As all these variables may be dysregulated in current smokers, the observations may partly explain why current smokers are reported to have an increased risk of many types of cancers.

Whether nicotine contributes or causes our observed associations is uncertain, as cigarette smoke contains numerous established carcinogens, and further tobacco-specific nitrosamines that produce reactive oxygen species [35] mediating multiple harmful effects including atherosclerosis. Thus, detailed effects of the cigarette smoke need to be focused to identify which are addressing the different components.

As shown from the Online Supplementary Table 2, angiotensin II receptor blocker and insulin were more frequently used in the non-smoking group as compared to current smokers, and we also observed that these drugs induced elevated levels of circulating IL-12 and mir-21. As patients with CAD commonly are treated with these drugs, this is worth to be noticed. Aspirin

and angiotensin converting enzyme (ACE) -inhibitors have previously been reported to suppress IL-12 production in leukocytes [36;37]. In the present investigation, all patients were on aspirin treatment at time of inclusion and ACE-inhibitors were not differently distributed according to smoking groups and not associated with modified IL-12-levels, thus not corrected for.

Limitations

Mir-21 regulates multiple genes and IL-12 may as well be regulated by other miRNAs. Additionally, miR-21 is most likely under strict regulation by other regulatory mechanisms i.e. long non-coding RNA molecules, which are capable of binding up excessive amounts of miRNAs preventing them from inhibiting their mRNA targets. One such example is growth arrest-specific 5 (GAS5) [38]. In light of miRNAs' promiscuity and complexity concerning their function, along with small numbers in the sub-group analysis, the present study should be regarded as experimental.

Conclusion

In conclusion, circulating mir-21 and expression of the IL-12p35 gene were observed significantly downregulated in current smokers with CAD. The results may indicate miR-21 as a modest regulator of IL-12p35mRNA, as the variables seem to mirror each other rather than being inversely correlated, hence the nexus of cause and consequence needs to be explored. The IL-12/INFγ pro-inflammatory axis seemed further to be potentiated, although dampened, by cigarette smoking, suggesting an altered regulation of this pathway in current smokers. Finally, influence of the IL-12p40 3'UTR 1188 A/C polymorphism on circulating IL-12 levels may have distinct clinical impacts in smokers as compared to non-smokers. In light of IL-12's role in immunity and anti-tumor activity, the influence of smoking on the markers needs to be further investigated.

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Conflicts of Interest: None

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Supplementary Table 1: Demographic data present in the cohort analyzed for IL-12p35 and INFγ gene-expression, as related to smoking.

	Non-smokers (n = 234)	Current smokers (n = 96)	p-value
Age (years, mean (range))	63 (37-81)	58 (39-75)	**>0.001**
Men/Women n (%)	187/46 (80/20)	73/23 (76/24)	ns
Type 2 Diabetes n (%)	56 (24)	16 (17)	ns
Hypertension n (%)	132 (56)	41 (43)	**0.021**
Metabolic Syndrome	72 (31)	35 (34)	ns
BMI (kg/m$^{2)*}$	27.4 (25.0, 30.0)	27.2 (24.3, 30.2)	ns
CRP (mg/L)*	1.80 (0.90, 3.00)	2.34 (1.43, 4.10)	**0.002**
Triglycerides (mmol/L)*	1.37 (0.94, 1.94)	1.48 (1.03, 2.10)	ns
Tot-C (mmol/L)	4.6 (0.9)	4.9 (1.1)	**0.012**
HDL-C (mmol/L)	1.3 (0.4)	1.3 (0.4)	ns
Fasting glucose (mmol/L)	6.2 (2.2)	6.1 (2.2)	ns

Values are mean (SD) or number (proportions) if not otherwise stated. SD: standard deviation, BMI: body mass index, CRP: C-reactive protein, Tot-C; total cholesterol, HDL-C: high-density Lipoprotein cholesterol.

p-values refer to differences in variables between non-smokers and current smokers

* Median levels (25, 75 percentiles)

Supplementary Table 2: Demographic data present in the total ASCET cohort, as related to cigarette smoking

	Non-smokers (n = 798)	Current smokers (n = 203)	p-value
Age (years, mean (range)	63 (36-81)	58 (39-78)	**<0.001**
Men/Women n (%)	635/162 (81/75)	148/55 (19/25)	**0.037**
Type 2 Diabetes n (%)	165 (21)	35 (17)	ns
Hypertension n (%)	464 (58)	92 (45)	**<0.001**
BMI (kg/m^2)*	27 (25, 29)	27 (25, 30)	ns
CRP (mg/L)*	1.7 (0.90, 3.00)	2.6 (1.40, 4.45)	**<0.001**
Triglycerides (mmol/L)*	1.27 (0.91, 1.82)	1.45 (1.03, 1.92)	**0.002**
Tot-C (mmol/L)	4.5 (1.0)	4.8 (1.0)	**0.001**
HDL-C (mmol/L)	1.4 (0.4)	1.2 (0.9)	**0.001**
Fasting Glucose (mmol/L)	6.1 (1.9)	6.0 (1.9)	ns
Medication (%)			
Aspirin	798 (100)	203(100)	ns
Statins	782 (98)	200 (99)	ns
β-blockers	605 (76)	152 (75)	ns
CCB	209 (26)	46 (23)	ns
Nitrates	172 (22)	45 (22)	ns
ACE inhibitors	214 (27)	49 (24)	ns
ARB	204 (26)	35 (17)	**0.012**
Diuretica	180 (23)	46 (23)	ns
Insulin	40 (5)	4 (2)	0.06
Oral antidiabetics	97 (12)	21 (10)	ns

Values are mean (SD) or number (proportions) if not otherwise stated. SD: standard deviation, BMI: body mass index, CRP: C-reactive protein, Tot-C; total cholesterol, HDL-C: high-density

lipoprotein cholesterol, ACE: angiotensin converting enzyme, ARB: angiotensin II receptor blocker, CCB: calcium channel blocker.

p-values are chi-square test for categorical variables and *t*-test and Mann-Whitney test for continuous variables, referring to differences between non-smokers and current smokers.

* Median levels (25, 75 percentiles).